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Muscarinic receptor-mediated intracellular Ca^{2+} mobilization in embryonic chick heart cells

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Activation of muscarinic receptors of heart cells elevates the intracellular Ca^{2+} concentration. The increase is considered to be due to influx of extracellular Ca^{2+}. We show that intracellular Ca^{2+} mobilization is involved. Cell suspensions prepared from hearts of 6-day-old chick embryos were loaded with the fluorescent Ca^{2+} chelator chlortetracycline. Muscarinic stimulation induces a dose-dependent fluorescence decrease ($ED_{50} = 2.6 \times 10^{-6}$ M) indicating intracellular Ca^{2+} mobilization.

Ca^{2+} mobilization; Muscarinic receptor; Chlortetracycline; (Chick heart)

1. INTRODUCTION

Activation of cardiac muscarinic receptors results in increased K^+ permeability [1,2], decreased adenylate cyclase activity [3,4], increased cyclic GMP formation [5] and stimulation of phosphoinositide turnover [6–8].

In most tissues and cells, phosphoinositides are hydrolysed to give diacylglycerol and phosphatidylinositol phosphates [9] from which inositol trisphosphate triggers intracellular Ca^{2+} mobilization [10,11].

In the heart, stimulation of phosphoinositide turnover [6–8] and synthesis of IP_3 [12] by muscarinic agonists have been demonstrated. However, there is still controversy about the effect of IP_3. Hirata et al. [13] were able to demonstrate Ca^{2+} release from the sarcoplasmic reticulum of canine ventricular muscle after addition of IP_3, whereas Movsesian et al. [14] did not find Ca^{2+} release on permeabilized cardiac myocytes and sarcoplasmic reticulum of the rat.

High concentrations of muscarinic agonists have a positive inotropic effect which might be due to increased intracellular Ca^{2+} concentration [15–17]. An increased intracellular Ca^{2+} level after stimulation of muscarinic receptors has recently been demonstrated in rat ventricular myocytes with quin2 [18]. The increase in intracellular Ca^{2+} concentration was accompanied by an increase in intracellular Na^+ and was dependent on extracellular Na^+ and Ca^{2+}. In addition, the increase was independent of intracellular Ca^{2+} mobilization because inhibiting intracellular Ca^{2+} mobilization with ryanodine and discharging Ca^{2+} stores with caffeine were without effect on the quin-2-measured Ca^{2+} increase. The authors therefore assume that the increase in intracellular Ca^{2+} is caused by influx of extracellular Ca^{2+} in exchange for intracellular Na^+ which increases after muscarinic stimulation [15,16,18].

In contrast to the above-cited experiments we show with the fluorescent Ca^{2+} chelator chlortetracycline that in chick embryonic heart cells activation of muscarinic receptors leads to intracellular Ca^{2+} mobilization.

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Abbreviations: IP_3, *myo*-inositol 1,4,5-trisphosphate; CTC, chlortetracycline

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2. MATERIALS AND METHODS

Suspensions of 6-day-old chick embryonic heart cells (stage 29) were prepared by collagenase/hyaluronidase (Boehringer) treatment as in [19] for the isolation of chick limb bud cells. Isolation was performed at room temperature. The isolated cells were kept in Hanks solution at 25°C until use.

Intracellular Ca^{2+} mobilization after muscarinic stimulation was measured by CTC fluorescence. The procedure is described in detail by Oettling et al. [20]. A 2 ml aliquot of cell suspension (4×10^6 cells/ml) was preincubated with 20 μM CTC for 25 min in modified Hanks (10 mM Hepes, 2.1 mM $NaHCO_3$). The cell suspension was supplemented with 1×10^{-5} M eserine in order to inhibit cholinesterase.

Acetylcholine, added in 2-μl units to the stirred cell suspension, triggered a fluorescence decrease. The reaction had reached completion 4 min after addition of acetylcholine. The fluorescence change was determined graphically and corrected for dilution produced by the volume added as in [20]. The relative fluorescence change was calculated as the percentage of the maximal inducible fluorescence decrease. The parameters of the dose-response curves were fitted to the function

$$E = E_{max}[A]/ED_{50} + [A]$$

where E denotes the effect, E_{max} the maximal effect, ED_{50} the concentration of drug giving half-maximal effect and [A] the concentration of drug. Parameters were fitted with the non-linear least-squares regression programme BMDPAR (BMDP statistical software).

3. RESULTS AND DISCUSSION

CTC is known to form fluorescent complexes with Mg^{2+} and Ca^{2+}. Cellular CTC fluorescence is mainly caused by CTC molecules complexed to membrane-associated Ca^{2+} and Mg^{2+} [21]. The fluorescence intensity depends on the concentration of free Ca^{2+} in the storage vesicles [22,23]. Thus, intracellular Ca^{2+} mobilization is accompanied by a decrease in CTC fluorescence.

Fig.1 shows that acetylcholine triggers a concentration-dependent decrease in fluorescence in cell suspensions of embryonic heart cells. 3×10^{-6} M atropine prevents the effect of acetylcholine.

We recorded the excitation spectra of CTC-labeled cells before and after stimulation with acetylcholine (fig.2). As shown in [19], changes in CTC-Ca^{2+} fluorescence should be reflected by a peak at 397 nm in the difference spectrum and in CTC-Mg^{2+} fluorescence by a peak at 383 nm. The difference spectrum of chick heart cells (fig.2) peaks at 397 nm indicating that changes in CTC-Ca^{2+} fluorescence are responsible for the

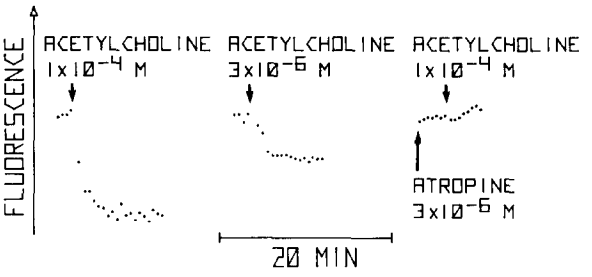

Fig.1. Effect of acetylcholine on CTC fluorescence of chick embryonic heart cells. On-line plots of fluorescence intensity of representative experiments. Cells were preincubated with 20 μM CTC for 25 min at 37°C in the dark and then transferred to a thermostatted fluorometer cuvette (37°C). Drugs were added (arrow) to the stirred cell suspension in a volume of 2 μl.

fluorescence decrease after addition of acetylcholine.

Fig.3 shows the dose-response curve of acetylcholine for Ca^{2+} mobilization. The ED_{50} was calculated to be 2.6×10^{-6} M ($2.4–2.8 \times 10^{-6}$ M; asymptotic SD; BMDP manual).

In embryonic chick heart cells muscarinic agonists are 100-fold more potent at inhibiting cyclic AMP formation than at stimulating phosphoinositide turnover [24]. The concentrations of muscarinic agonists for inducing the positive inotropic effect and the Ca^{2+} increase measured by quin2 fall within the range where phosphoinositide turnover is stimulated ($>10^{-6}$ M) [15–18]. The concentrations of acetylcholine

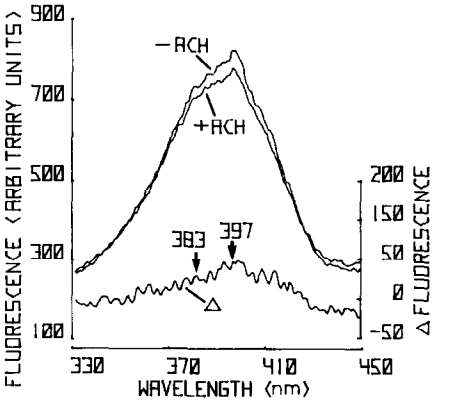

Fig.2. Excitation spectra. A cell suspension was scanned before (−ACH) and after (+ACH) stimulation with 1×10^{-4} M acetylcholine. Emission wavelength 529 nm. The difference spectrum (Δ) was calculated from the data of these spectra.

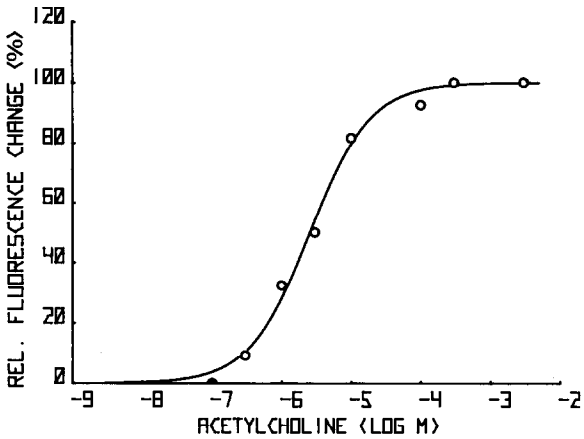

Fig.3. Concentration dependence of the acetylcholine effect. The results are taken from a single experiment. The experiment was repeated 3 times with essentially identical results.

necessary for triggering intracellular Ca^{2+} mobilization are in the same range. We therefore assume that the intracellular Ca^{2+} mobilization is triggered by IP_3.

The CTC method is not appropriate for investigations on the influx of Ca^{2+} into cells. Therefore, our experiments do not exclude the possibility of concomitant influx of Ca^{2+} into the cells. Both phenomena, influx of extracellular Ca^{2+} and Ca^{2+} mobilization out of intracellular vesicles, may contribute to the increased intracellular Ca^{2+} concentration after muscarinic stimulation of heart cells.

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